Research Article

The Metabonomic Studies of Tongue Coating in H. pylori Positive Chronic Gastritis Patients

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In Traditional Chinese Medicine (TCM), tongue diagnosis (TD) has been an important diagnostic method for the last 3000 years. Tongue coating can be used as a very sensitive marker to determine the progress of chronic gastritis. Therefore, the scientific, qualitative, and quantitative study for the pathophysiologic basis of tongue coating (TC) emerged as a major direction for the objective research of TD. In our current report, we used GC/MS technology to determine the potential changes of metabolites and identify special metabolic biomarkers in the TC of H. pylori infected chronic gastritis patients. Four discriminative metabolites were identified by GC/MS between the TC of H. pylori infection (G + H) and without H. pylori infection (G − H) patients: ethylene, cephaloridine, γ-aminobutyric acid, and 5-pyroglutamic acid, indicating that changes in amino acid metabolism are possibly involved in the formation of TC, and the amino acid metabolites are part of the material components of TC in G + H patients.

1. Introduction

Helicobacter pylori (H. pylori, Hp) infection is one of the most important causes of chronic gastritis and gastric cancer [1, 2]. Hp, a Gram-negative bacterium found in the stomach, is listed as Class I carcinogen by WHO. In 1984, Hp was first isolated from the gastric mucosa and epithelial surface by Marshall and Warren [3]. Hp infection can lead to chronic gastritis, gastric and duodenal ulcers, and increased risk of gastric cancer [1, 4–7]. Correa delineated the whole pathological process from Hp infection induced inflammation of gastric mucosa, to intestinal metaplasia, aplasia, and carcinoma [8]. In 1998, Watanabe et al. established the first animal model with Mongolian gerbils to demonstrate that Hp infection directly causes gastric cancer [9]. Our previous studies illustrated that Hp can grow in the stomach mucosa of C57BL/6 mice following oral gavage of the bacteria. Seventy-two weeks later, pathological examinations clearly revealed a 22.2% rate of gastric cancer incidence in the mice [10]. This piece of evidence again reaffirms the role of Hp in inducing gastric cancer; however, the mechanism remains elusive, is thought to be very complex, and involved numerous metabolic pathways in the body. This poses a huge challenge for the prevention and treatment of Hp induced chronic gastritis and gastric cancer. Among the many current studies of metabolic pathways and the metabolites in Hp induced chronic gastritis and cancer, Shi revealed that, in the serum samples of Hp infected patients, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) is significantly lower while the malonaldehyde (MDA) level is higher relative to Hp negative patients [11].

Tongue diagnosis is a noninvasive, simple, and valuable diagnostic tool, the use of which has been repeatedly affirmed by clinical practitioners of traditional Chinese medicine (TCM) for 3,000 years. Tongue appearance is closely associated with the physiology as well as pathophysiology of
the digestive system [12–14]. TCM theories state that the
tongue coating (TC) is condensed stomach “Qi” and the
essence “Qi” of food; tongue appearance is a very sensitive
index of the physiological and pathological status of the
organs, especially the stomach and spleen. Tongue appear-
ance reflects the amount of bad “Qi” and the dynamic process
of illness of the stomach, that is, the Hp infection status.
Clinical research has reported that the tongue appearance
changes provide essential information for the diagnosis,
treatment, and prognosis of chronic gastritis, peptic ulcers,
and gastric and colorectal cancers [15–18]. Huang et al.
discovered that, in the patients with Hp induced chronic
superficial gastritis, the colors of TC were mainly light white
and yellow [19]. This finding was corroborated by another
report by Wang et al., which suggests that, in 518 chronic
gastritic cases, 81.6% of Hp-infection positive patients had
yellow CT, significantly higher than the Hp negative group
[20]. Mao reported that the tongue color of the majority of Hp
positive patients was light red while the TC appeared greasy,
thick, and yellow [21]. Together, these and other studies have
demonstrated that tongue appearance correlates with Hp
infection status: positive patients have red or purple tongues
with yellow TC, and the more severe the Hp infection gets, the
thicker and greasier the TC appears. Tongue appearance also
reflects the degrees of gastric inflammation and prognostic:
when TC turns to be thinner, it indicates a better function
status of stomach and spleen, less inflammation, and less Hp
infection [22].

Metabonomics is an important part of the system biology.
Metabolites are the ultimate products of gene expression,
closely related to the physiology and pathophysiology of the
body. Metabonomics considers the human body as a whole
system, which is consistent with the TCM concept, and
therefore has wide application prospects in TCM research
[23–25]. The analysis of syndrome (“zheng” in TCM) asso-
ciated metabolites may help comprehend the changes of met-
abolic pathways and conditions when a disease progresses
and understand the material basis of the disease. Chen
et al. reported a specific metabolite, 1-methyladenosine,
as biomarker in hepatocellular carcinoma patients using
metabonomics [26]; Leichtle et al. investigated the levels of
26 amino acids in the blood of colorectal cancer patients
and found that the cancer patients had lower concentration for
11 amino acids and proposed a carcinoembryonic antigen-
(CEA-) glycine-tyrosine tri-biomarker, the best model for the
diagnosis of the disease [27]. Chronic gastritis is associated
with Hp infection and TC is a reliable status indicator of
Hp infection, gastric inflammation, and prognosis. Hence, in
the current study, we used GC/MS technology to investigate
the spectrum of material composition in TC of Hp infected
patients, determine the changes of TC metabolites, and iden-
tify microorganism biomarkers for the Hp positive, chronic
gastritis patients.

2. Materials and Methods

2.1. Ethical Statement. All samples were obtained as part
diagnostic criteria after patients gave written informed

<table>
<thead>
<tr>
<th>Group</th>
<th>Tongue coating</th>
<th>Gender</th>
<th>Age (X ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp positive</td>
<td>2 13 8</td>
<td>Male 12</td>
<td>51.71 ± 13.42</td>
</tr>
<tr>
<td>Hp negative</td>
<td>9 1 9</td>
<td>Female 7</td>
<td>58.57 ± 10.69</td>
</tr>
</tbody>
</table>

consent. The study was approved by the local ethics commit-
tee of Shanghai University of Traditional Chinese Medicine
Shuguang Hospital (SUTCMSH), Shanghai, China.

2.2. Participant Selection Criteria and TC Samples Details.
The participants of this study were mainly patients from
SUTCMSH, from October 2012 to July 2013. All patients
underwent a gastroscopy examination for diagnosis of
chronic gastritis and a gastric mucosa biopsy and Giemsa
staining to confirm Hp infection. Twenty-nine patients had
both chronic gastritis and Hp infection while 13 patients
had only chronic gastritis. Our study included 42 chronic
gastritis patients at Shuguang Hospital, Shanghai University
of Traditional Chinese Medicine, between October 2012 and
July 2013. Of the 42 cases, 23 were positive of Hp infection
(12 male, 11 female), with the mean age of 51.71 ± 13.42 years; 19
were negative of Hp infection (7 males, 12 females), with the
mean age of 58.57 ± 10.69 years. The TC color in the Hp group
was mainly yellow while mainly white or white/yellow in the
non-Hp group (Table 1). No significant differences in gender
and age were observed between the two groups (P > 0.05).

2.3. Tongue Coating (TC) Samples Collection. The TC samples
were collected as previously described [22]. All participants
were required to gargle saline 2–3 times before sampling to
rinse possible food contamination that might influence the
TC. Small spoons were used to scrape the TC at the thickest
area and samples were placed into sanitized Eppendorf tubes
that had been filled with 2 mL of sterile saline. All samples
were stored at −80°C until analysis (Figure 1).

2.4. Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

2.4.1. GC/MS Measurement. TC samples were prepared by
sonication and centrifugation at 4°C 3500 rpm for 10 min. The
100 μL supernatant was transferred to a new tube and after
adding 200 μL methanol, it was vortexed for 30 s, incubated
at 20°C for 10 min, and centrifuged at 4°C 10000 rpm for
10 min; then 200 μL supernatant was transferred to a sample
tube. Sample was then freeze-dried, added 10 μL of chlor-
ophenylalanine (0.3 mg/mL) and 30 μL of methoxamine
pyridine (15 mg/mL), sealed, vortexed for 30 s, incubated
at 37°C for 90 min, added 40 μL BSFTA (containing 1% TMCS),
incubated at 80°C for 2 h, cooled at room temperature for 1 h,
before being analyzed by GC/MS. The GC/MS system was
from Agilent Technology (California, USA), Model# DB5MS,
column: 30 m × 0.25 mm × 0.25 μm. GC/MS condition is
as follows: the column temperature was held at 80°C for
3 min, then 10°C/min increased to 140°C, 4°C/min increased
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The TC samples of *Hp* positive and *Hp* negative chronic gastritis patients demonstrated a clear difference between the two groups (Figure 2). In order to determine the detailed metabolomic profiles, multivariate statistical analysis was performed for the samples, that is, the principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares-discriminate analysis (OPLS-DA).

3.2. The Metabonomics of TC Samples from the *Hp* Positive and *Hp* Negative Chronic Gastritis Patients. PCA scores of the TC samples from the two groups showed that all the sample points fell in the 95% confidence intervals but appeared partially overlapped, indicating that this method was not able to discriminate between the groups (Figure 3(a)). Further research by PLS-DA showed that the sample points were clearly separated (obtaining good class separation value and predictive power, with $R^2_Y = 0.82$), which indicated that the two groups' metabolic pathways were different: all the sample points of *Hp* positive patients were mainly in the left lower quadrant, while the *Hp* negative patients' sample points are in the right upper quadrant (Figure 3(b)). To improve the accuracy of the PLS discriminated model, OPLS–DA by SIMCA-P* software was used to analyze the results to better highlight the difference between the groups. The analysis result showed that the sample points from the two groups were completely separated in different quadrants: the *Hp* positive sample points were in the left quadrant and *Hp* negative in the right (Figure 3(c)).

3.3. The Different Metabolite Markers of TC Samples from the *Hp* Positive and *Hp* Negative Chronic Gastritis Patients. We used OPLS-DA to block out irrelevant signals, to acquire reliable metabolite marker peaks. The metabolites responsible for discrimination were selected according to the Variable Importance in the Projection (VIP) considering only variables with VIP values higher than 1.0, indicative of significant differences among groups (Table 2). These potential metabolite markers, identified using the NIST Mass Spectral Library and KEGG bioinformatics database, were G-aminobutyric acid, S-hydroxyproline, ethylene, and some amino acids (Table 3).

4. Discussion

As a unique method, the tongue diagnosis contributed a great deal for the formation and development of TCM theory system [28], “Huang Di Nei Jing,” an ancient TCM book written in Qin and Han era (∼2000 years ago), recorded the uses of tongue diagnosis. A chapter in that book called “Ling Shu, Shi Zhuan” stated the following: “By observing lip and tongue, one can determine the stages of a disease.” Tongue coating (TC), as the main part of the tongue appearance, is the moss or fur like material on the tongue surface. The TCM believes that the changes of TC reflect human body’s physiology and pathophysiology status. As described by “Xing Se Jian Mo”: “the TC is formed by stomach (“stomach-Qi” in Chinese) and the five organs (“Wu-Zang” in Chinese) are all supplied by

3. Results

3.1. Chromatographic Analysis and Comparison between the *Hp*-Infection Positive and Negative Chronic Gastritis Patients. The total ion chromatograms obtained by GC/MS from to 240°C, 10°C/min increased to 280°C, and it was held for 10 min. Injection inlet temperature was 280°C and sensor temperature was 300°C. The carrier gas is Helium and flow rate was 1 mL/min. MS condition is as follows: EI ionization, electron energy 70 eV, ion source temperature 250°C, interface temperature 250°C, solvent delay 5 min, full-spectral scan, and scan scope M/Z 40–600.

2.4.2. GS/MS Data Analysis. Raw data was processed through multiple stages including noise reduction, feature detection, alignment of peaks, and normalization. GC/MS data were analyzed by Agilent Mass Profiler Professional (MPP) software. We analyzed the previously processed data by SIMCA-P* software (V13.0, Umetrics AB, Umea, Sweden), principal component analysis (PCA) is used to analyze the data by Centered Scaling method, and the data is automatically modeled and analyzed; partial least squares-discriminate analysis (PLS-DA) is used to analyze the data by Centered Scaling method, and the data is automatically modeled, modeling analysis of the first and two principal components; and orthogonal partial least squares-discriminate analysis (OPLS–DA) showed the maximum differences between different groups within the model.

2.4.3. Identification of Metabolite Markers. Differential metabolites markers were selected according to the PLS-DA Variable Importance in the Projection (VIP), considering only variables with VIP values higher than 1, indicative of significant differences among groups. These potential markers were identified by retention time correction of peaks and mass-to-charge ratio (m/z) using the Mass Spectral Library (National Institute of Standards and Technology, NIST).

![Figure 1: Sampling images of tongue coating from the center of the tongue, an area regarded as tongue coating in the traditional tongue diagnosis.](image-url)
the stomach, so the TC is the index of body status.” “New Ling Shu” explained: “Tongue is closely related to the digestive system; whenever the digestive organs have problem, TC will show it.” Therefore, not only can TC be an indicator of the pathophysiological status of the five essential organs, but also a “window” for the development stages of the gastric illnesses, a sensitive index for the progression of chronic gastritis [29]. In summary, to investigate the underlying mechanisms of TC formation, we can explore the nature of chronic gastritis TCM syndromes and obtain new clues and novel ideas for the objective studies of TCM syndromes.

*Hp* infection, one of the most causative factors of chronic gastritis and gastric cancer, has been listed as Class I carcinogen by WHO cancer institutions. Wang et al. reported that, in 518 chronic gastritis patients, 440 cases were *Hp*-infection positive (85%) and mainly had yellow TC (81.16%), significantly higher than the *Hp* negative group [20]. The reason for the yellow appearance of the TC was probably due to *Hp* infection increased gastric inflammation, which led to the malfunctioning of digestive system, lowered saliva secretion, and decreased oral cavity self-cleaning. This resulted in tongue surface dysbacteriosis that caused inflammation, exudate, and yellow-color change of the tongue. This is just a hypothesis, which apparently needs to be studied further and supported by experimental evidence. Therefore, how the *Hp* infection causes TC changes still remains an unsolved problem.

Metabolomics, based on the analysis of the entire set of metabolites in a sample, provides a comprehensive overview of the status of organisms, more directly and accurately reflecting the pathophysiology of the organisms. Biomarkers discovery is the current research “hotspot,” but most of the metabolite biomarkers are identified in blood, urine, and tissue samples, rarely in TC samples. TC metabolomics, the study of the metabolites of TC samples to determine the pathophysiology status of the human body, has recently emerged. Li et al. established the methodology to process TC sample for metabolomics analysis [30]. Sun et al. discovered 10 discriminative metabolite biomarkers between TC samples of normal and chronic gastritis groups, using LC/MS technology [25]. Zhao et al. utilized GC/MS technology to uncover 17 metabolite biomarkers between normal and chronic hepatitis groups [31]. TC, as biological sample, is convenient and noninvasive to collect and is unique to TCM,
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Tough (PCA-X)

Tough (PLS-DA)

Tough (OPLS-DA)

Figure 3: The metabonomics of tongue coating samples from Hp positive and Hp negative chronic gastritis patients. (a) PCA analysis, (b) PLS-DA analysis, and (c) OPLS-DA analysis.

which believes that TC is condensed Qi and liquid (“Jin” in Chinese) evaporated from the spleen and stomach on the tongue surface, so TC reflects the physiological and pathological status of the human body. Our current report researched TC samples to determine metabolite biomarkers in the TC of the Hp infection induced chronic gastritis patients.

We used PLS-DA and OPLS-DA statistical methods to analyze the GC/MS data of TC samples from Hp positive and Hp negative chronic gastritis patients and found a difference between the metabolites of each group. Using the NIST Mass Spectral Library and KEGG bioinformatics database, we identified these discriminative metabolite biomarkers as γ-aminobutyric acid, 5-hydroxyproline, ethylene, and pyroglutamic acid which is derived from glutamine through dehydration and cyclization. Glutamine is one of the 20 common amino acids of the human body. It can form glutathione (GSH) by synthetically reacting with cysteine and glycine [32]. GSH plays a role in the biodefense system of the human body, that is, proimmunity, antiaging, and detoxicating. The TC samples of the Hp positive chronic gastritis patients had higher amount of pyroglutamic acid (VIP > 1), which indicates that the synthesis pathway of GSH was blocked, as the glutamine was not used to make GSH but directed toward the dehydration/cyclization reaction, to form pyroglutamic acid. The imbalance of GSH metabolism will disrupt normal physiology, causing a decrease of immune and detoxicating functions of the human body. McNulty and Dent uncovered that highly homogeneous groups of
Table 2: Comparison of chromatogram peaks from the TC samples of \( H_p \) positive and \( H_p \) negative chronic gastritis patients.

<table>
<thead>
<tr>
<th>Var ID</th>
<th>( m/z )</th>
<th>Mean peak area (( H_p ) positive)</th>
<th>Mean peak area (( H_p ) negative)</th>
<th>( P ) value</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>593</td>
<td>73</td>
<td>0.000124052</td>
<td>0.000290037</td>
<td>0.04129</td>
<td>2.0532</td>
</tr>
<tr>
<td>64</td>
<td>73</td>
<td>0.000231336</td>
<td>8.16838E-05</td>
<td>0.04928</td>
<td>2.05431</td>
</tr>
<tr>
<td>512</td>
<td>73</td>
<td>0.000162672</td>
<td>2.74211E-05</td>
<td>0.03997</td>
<td>2.09457</td>
</tr>
<tr>
<td>623</td>
<td>73</td>
<td>7.71475E-05</td>
<td>0.000303819</td>
<td>0.04789</td>
<td>2.09683</td>
</tr>
<tr>
<td>42</td>
<td>43</td>
<td>0.001224892</td>
<td>0.000927157</td>
<td>0.05121</td>
<td>2.17178</td>
</tr>
<tr>
<td>11</td>
<td>73</td>
<td>0.022093242</td>
<td>0.037576638</td>
<td>0.04812</td>
<td>1.06903</td>
</tr>
<tr>
<td>668</td>
<td>73</td>
<td>0.000341666</td>
<td>5.39923E-05</td>
<td>0.05087</td>
<td>2.29584</td>
</tr>
<tr>
<td>799</td>
<td>73</td>
<td>0.000312782</td>
<td>0.004340828</td>
<td>0.04879</td>
<td>2.62218</td>
</tr>
<tr>
<td>680</td>
<td>73</td>
<td>0.000954827</td>
<td>0.000677735</td>
<td>0.04762</td>
<td>2.75245</td>
</tr>
<tr>
<td>595</td>
<td>73</td>
<td>0.000208521</td>
<td>5.79851E-5</td>
<td>0.03584</td>
<td>2.79777</td>
</tr>
<tr>
<td>523</td>
<td>73</td>
<td>5.86622E-05</td>
<td>0.000264136</td>
<td>0.04978</td>
<td>2.87228</td>
</tr>
<tr>
<td>321</td>
<td>57</td>
<td>0.000112378</td>
<td>9.7488E-05</td>
<td>0.05041</td>
<td>1.16753</td>
</tr>
</tbody>
</table>

Note: we selected the different materials between \( H_p \) positive and \( H_p \) negative patients with \( P < 0.05 \), VIP > 1.0.

Table 3: The potential metabolite biomarkers and related metabolic pathways in the TC of \( H_p \) positive and \( H_p \) negative chronic gastritis groups.

<table>
<thead>
<tr>
<th>Var ID</th>
<th>CAS1/NIST</th>
<th>CAS2/NIST</th>
<th>Name</th>
<th>KEGG ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>593</td>
<td>7381-30-8</td>
<td>—</td>
<td>Ethylene</td>
<td>C06547</td>
</tr>
<tr>
<td>64</td>
<td>1126-58-5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>512</td>
<td>50-59-9</td>
<td>—</td>
<td>Cephaloridine</td>
<td>C11754</td>
</tr>
<tr>
<td>623</td>
<td>39508-23-1</td>
<td>—</td>
<td>( \gamma )-Aminobutyric acid</td>
<td>GABA</td>
</tr>
<tr>
<td>42</td>
<td>1126-58-5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>668</td>
<td>30274-77-2</td>
<td>—</td>
<td>Pyroglutamic acid</td>
<td>C01879, C02237</td>
</tr>
<tr>
<td>799</td>
<td>54477-01-09</td>
<td>55521-23-8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>615</td>
<td>1126-58-5</td>
<td>50-59-9</td>
<td>Cephaloridine</td>
<td>C11754</td>
</tr>
<tr>
<td>680</td>
<td>1126-58-5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>595</td>
<td>55521-23-8</td>
<td>50-59-9</td>
<td>Cephaloridine</td>
<td>C11754</td>
</tr>
<tr>
<td>523</td>
<td>7381-30-8</td>
<td>1126-58-5</td>
<td>Ethylene</td>
<td>C06547</td>
</tr>
<tr>
<td>321</td>
<td>39508-23-1</td>
<td>—</td>
<td>( \gamma )-Aminobutyric acid</td>
<td>C00334</td>
</tr>
</tbody>
</table>

Note: CAS1/NIST is the potential metabolite biomarkers number in NIST Mass Spectral Library, and KEGG ID is the potential metabolite biomarkers number in KEGG bioinformatics database.

\( C. \ pylori \) produce a similar panel of enzymes, including oxidase, DNase, oxidase, catalase, urease, alkaline phosphatase, leucine aminopeptidase, and \( \gamma \)-glutamyl aminopeptidase [33]; therefore, our future research projects will be focused on interrogating whether the \( H_p \) produced \( \gamma \)-glutamyl aminopeptidase affects the metabolism of glutamine.

5. Conclusions

We used GC/MS technology to determine the metabolic components of tongue coating samples in chronic gastritis patients with or without \( H_p \) infection. We found distinct metabonomic differences between the 2 patient groups and identified 4 discriminative metabolite biomarkers in the tongue coating of \( H_p \) positive chronic gastritis patients: ethylene, cephalaridine, \( \gamma \)-aminobutyric acid, and 5-pyroglutamic acid. The discovery of these metabonomic biomarkers in the tongue coating not only can help the diagnosis and treatment of \( H_p \) infection induced chronic gastritis, but also provide a theoretical basis for the utilization of tongue coating aided clinical diagnosis of diseases.

Disclosure

Xuan Liu and Zhu-Mei Sun are co-first authors.

Conflict of Interests

The authors report no conflict of interests.
Authors’ Contribution

Xuan Liu and Zhu-Mei contributed equally to this work.

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